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Original Article

GEOGRAPHICAL IMPACTS OF PODOPHYLLOTOXIN (ANTI-CANCER DRUG AGENT) FOUND IN ROOTS OF PODOPHYLLUM HEXANDRUM & YIELD ENHANCEMENT USING PRECURSORS AND ELICITORS: LC-MS/MS METHOD

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ABSTRACT

Podophyllum hexandrum is a well-known medicinal plant, which demonstrates numerous therapeutic effects due to the presence of Podophyllotoxin, especially in Oncology. In this study, after development and validation of an RP-HPLC-MS method, levels of Podophyllotoxin in root samples of this plant collected from north of the Himalayas were measured, and environmental factors affecting its content were investigated. Five groups of plant populations from different spots of northern Himalayas were collected, and concentration levels of Podophyllotoxin were measured applying a validated High Performance Liquid Chromatography- Diode Array Detector/Mass Spectrometry (HPLC-DAD/MS) method. The impact of geographical parameters encompassing altitude and an average temperature of Podophyllotoxin contents was assessed. The validated method was selective, with good resolution, excellent linearity (r^2 > 0.9997), high accuracy, sensitivity and precision. The results illustrated that there was a direct correlation between altitude with the content of Podophyllotoxin in plant, which means that more the altitude, the more the content of Podophyllotoxin. In an opposite manner, levels of the Podophyllotoxin reversely correlated with the average temperature, in a way that, decreasing this variable resulted in raising the amount of the Podophyllotoxin. Treatments of cultured cells with fungal electors have been shown to induce the phenylpropanoid/flavonoid biosynthetic pathways and electors methyl jasmonate at 15 µM after 4 days resulted in higher HPLC content (8.606 %) and of isosafrole at 10 µM after 8 days shows 5.244%.

KEYWORDS: Podophyllotoxin, Podophyllum Hexandrun, LC-MS/MS, Environment, Altitude, Elicitors & Precursors

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INTRODUCTION

In India *Podophyllum hexandrum* is mostly found in Alpine Himalayas (2000-4000 m) of Jammu and Kashmir, Himachal Pradesh, Sikkim, Uttarakhand and Arunachal Pradesh [1].

Podophyllum plant species revealed presence of a number of compounds like pedophilia, Podophyllotoxin (PPT), querectin, 4-dimethypodophyllotoxin, kaempherol, picropodophyllotoxin, α-peltatin and β-peltatin [2]. The Podophyllotoxin content in Indian *Podophyllum* is more (7-15%) in comparison to other species, notably *Podophyllum peltatum* (4-8%), the most common species in American sub-continent [3]. Recently Podophyllotoxin has acquired great importance and high medicinal status due to its effectiveness as antimitotic, anticancer and immunostimulatory activity [4-6], especially for curing uterine tumors [7]. *Podophyllum hexandrum* extracts have been reported to offer radioprotection by modulating free radical flux involving the role of lignans presents [8]. Due to its anticancerous property, Podophyllotoxin is in increased demand throughout the world. Biosynthesis of Podophyllotoxin is an excessive process and availability of compound from natural resource is an important issue

for pharmaceutical companies that manufacture these drugs [9]. The annual supply is at present estimated at 80 tonnes, while the demand is more than 100 tonnes. To meet this ever increasing demand of crude drug, the roots (rhizomes) of *Podophyllum hexandrum* are being indiscriminately collected in large quantities [10]. As a result *Podophyllum hexandrum* is reported as an endangered species in Himalayan region and its preservation and quality assessment becomes even more important. The content of many secondary metabolites in plants can be affected by different factors encompassing ecosystem, location, climate, environmental parameters, and physical or chemical stresses etc. [11-13]. As a matter of the fact, the response of plants to these variables, depends on their ecophysiology and life history traits, and dramatically varies from one species to another. Ecosystem factors, such as latitude/longitude coordination, altitude, terrain and average temperature put crucial impacts on the content of metabolites in plants. Several studies have noted that when the climate and ecosystem characteristics vary, the production of metabolites is also altered [14-16]. Variations in climate and terrain affect the biosynthesis ability of *Podophyllum hexandrum* to produce Podophyllotoxin and other linens. The results of their study showed that regardless of ecosystem type, inhabitants of more northerly and westerly positions, and at lower elevations were associated with higher levels of secondary metabolites, and it was reported that phenolic compounds in the roots of *Glycyrrhiza glabra* can be altered as a result of changing climate variables [17]. Traditionally, developed methods with TLC, HP-TLC and NP-HPLC for Podophyllotoxin quantification from *Podophyllum hexandrum* are lengthy, time consuming and costly, leading to higher material cost and increased energy consumption, labor and even hazardous for nature with the use of several non-green solvent systems [18-20].

In this assessment, we determined concentration levels of Podophyllotoxin (PPT) in the roots of *Podophyllum hexandrum* populations collected from north of the Himalayas. The aim of our study was to develop a new LC-DAD/MS method [21-22] for PPT quantification and to inquest the influence of geographical variables with the contents of PPT in *Podophyllum hexandrum*. The results of this study will lead us to new insights for potent sources of this plant, linen and their optimum growing sites and its yield were enhanced using various precursors and electors.

MATERIAL AND METHODS

Chemicals and Solutions

Acetonitrile (Merck, HPLC grade), HPLC grade water (Ranbaxy, Mumbai), Chloroform, n-Hexane, Ethyl acetate, Ethanol, 25% NH_4OH, 0.1% HCOOH, 10mM NH_4OAC in water, all Chemicals were of Analytical Grade. The Standard Podophyllotoxin was purchased from Sigma-Aldrich chemicals and was at least of 99% purity.

Sample Collection from Plant Natural Ecosystem

Plant materials (Roots of *Podophyllum hexandrum*) for our study were collected from different altitudes in India during the months of November (2014), January and June (2015) and the exact geographical location of the collection stations were determined and recorded as per Sate Govt. Reports. Podophyllum parts were tested for identity at UHF, H.P and determined that they have similar morphology with the same age.

Extract Preparation

Parts of *Podophyllum hexandrum* collected from different regions were grinded under liquid nitrogen after air drying in shades. Two grams of each sample powdered air were put in 20 ml of falcon tube with methanol, vortexed for 30 minutes. Kept the tubes as such overnight, centrifuged at 4000 rpm for 10 minutes at room temperature and then collected the methanolic supernatant. The step was repeated several times to get the bulk material and to avoid the wastage.

HPLC-DAD Quantitative Analysis

Podophyllotoxin's quantitative analysis was performed by High performance liquid chromatography equipment consisted of a 1200 series binary pump (G1312B), a 1200 series gradient pump (G1310A) and a degasser (G1379B) (Agilent, Technologies, Germany) connected to an auto sampler with chemstation 6.0 version software package. Gradient chromatographic separation of Podophyllotoxin was performed on a SeQuant TM ZIC®-HILIC HPLC column along with an Oasis MCX 96-good solid-phase extraction cartridge with 40 min run time gradient [C-8: 4.0 mm × 175 mm, 1.5 µm particle size]. The injection volume was 20µL and the column oven temperature was set to 22°C. CH_3CN/water mobile phase combination (0.1% HCOOH to stabilize *p*H) was performed in gradient mode and re-equilibration for 5 minutes. The flow rate was optimized and set to 1.0 ml/min.

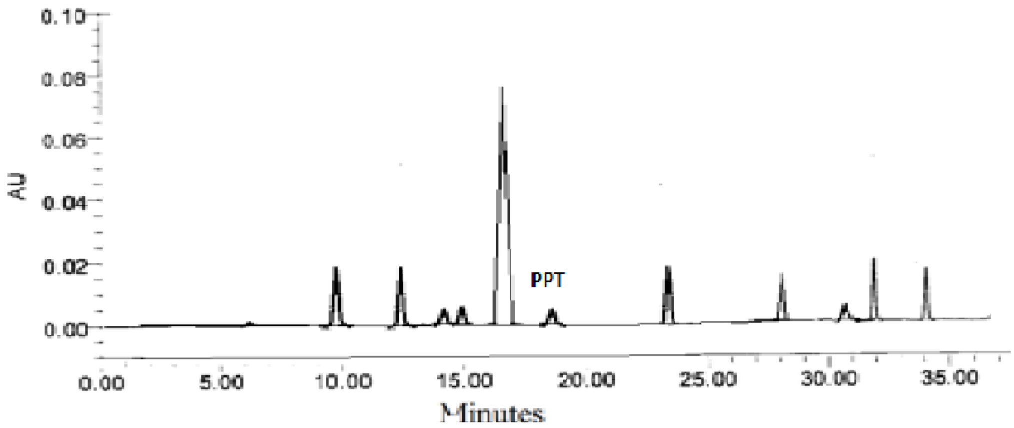

Figure 1: HPLC Chromatogram for the *Podophyllum Hexandrum* Root Extract, RHP Sample

[Retention Time, RT (PPT) = 19.2 minute]

APCI-3Q-MS/MS Profiling of Podophyllotoxin

MS/MS studies were performed on triple, quadruple, APCI-3Q-EMD/MS and Q Trap 5500 Mass Spectrometer. To achieve a fast separation, we used in fusion, MS sample introduction method for the APCI source. A hybrid triple quadrupole linear ion trap mass spectrometer ACPI 5500 Q-Trap equipped with a Turbo V source ion spray operating in positive and Negative modes was used for detection (Agilent, Germany). Nitrogen of 99% purity was generated and used. To minimize contamination of the mass spectrometer, the column flow was directed only from 2.0 to 5.0 min into the mass spectrometer using a diverter valve. Otherwise $CH_3CN$with a flow rate of 2.50µL/min was delivered into the mass spectrometer. The Turbo Ion Spray source was operated in the positive ion mode using the following settings: ion spray voltage =−4500 V, ion source heater temperature = 750 ^{o}C, source gas 1 = 45 psi, source gas 2 = 40 psi and curtain gas setting = 25 psi. Analyses were monitored in the multiple reaction monitoring (MRM) modes, mass transitions and MS parameters. Quadruples Q1 and Q3 were working at unit resolution.

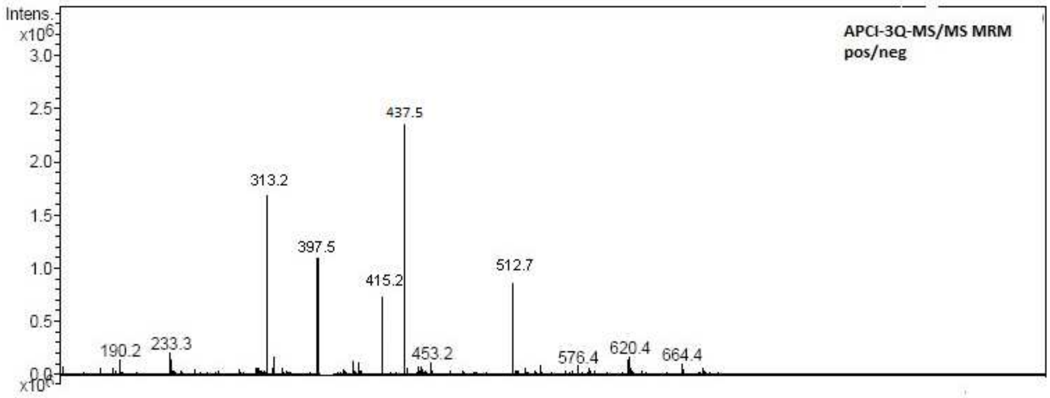

Figure 2: APCI-3Q-MS Spectra for the *Podophyllum Hexandrum* Showing PPT-Na Adduct

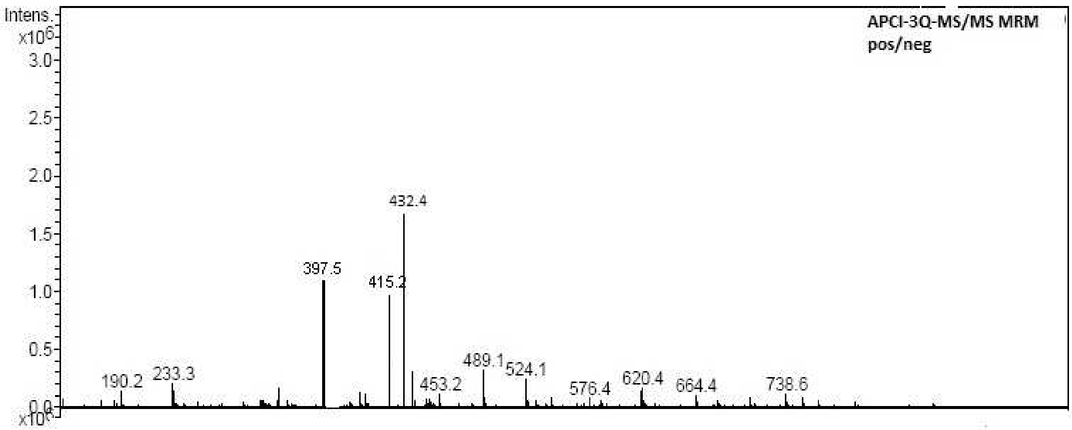

Figure 3: APCI-3Q-MS Spectra for the *Podophyllum Hexandrum* Showing PPT-H_2O Adduct

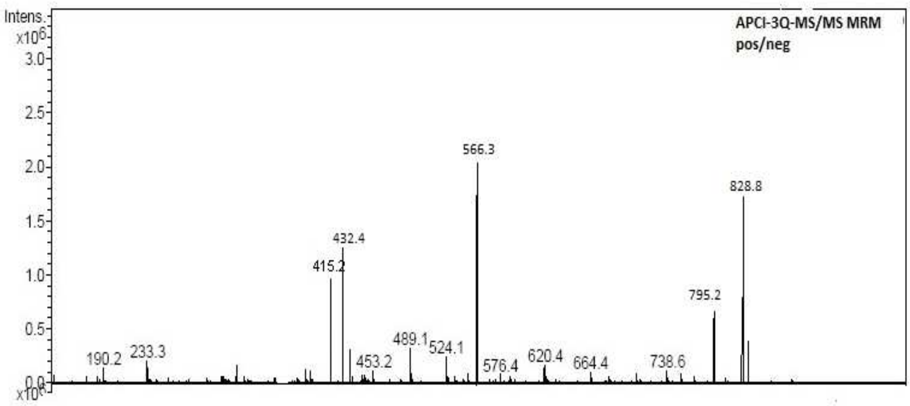

Figure 4: APCI-3Q-MS Spectra for the *Podophyllum Hexandrum* Showing PPT Dimer

Validation of the Method

The HPLC method was validated in terms of precision, accuracy, and linearity according to ICH guidelines. The accuracy of the assay method was evaluated in triplicate. The precision of the intra- and inter-day was evaluated by the repeated injection. Robustness of the method was demonstrated by changing the flow rate and wavelength. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting serial dilutions of solutions of the

standards with known concentrations [20].

Enhancement of Podophyllotoxin Yield by Using Precursors and Elicitors

Precursors of biosynthetic pathways have been used in plant cell suspension cultures to improve the production of secondary metabolites. Factors such as the concentration and the time of the addition of the precursor are to be considered, when applying the precursor to the cell culture medium. The addition of loganin, tryptophan and tryptamine enhanced the production ofsecologanin and indole alkaloids by *Catharanthus roseus* suspension cultures. Paclitaxel yields in the cell culture of *Taxus cuspidata* were improved up to six times by feeding phenylalanine and other potential paclitaxel side-chain precursors (*e.g*. Benzoic acid, *N*-benzoylglycine and serine). Two types of precursors ferrulic acid and eugenol with different type of concentration and time intervals were used to enhance the production. The addition of precursors ferrulic acid in the suspension culture improved HPLC purity to 5.845 % at 10 µM after 8 days and of eugenol at 15 µM after 16 days it was 4.188%.

Elicitation of secondary metabolite production in plant cell cultures could be induced either by biotic or abiotic molecules. Furthermore, electors stimulated the antioxidant defence systems of plant cells. Treatments of cultured cells with fungal elicitors have also been shown to induce the phenylpropanoid/flavonoid biosynthetic pathways. And, electors methyl Jasmonate at 15 µM after 4 days resulted in higher HPLC purity (8.606 %) and of isosafrole at 10 µM after 8 days shows 5.244%.

Table 1: Summary of Validation Parameters of RP-HPLC Method

Sr. No	Parameters	Experimental Value
1	Linearity and Range µg/ml	10-80
2	Correlation coefficient	0.9997
3	Accuracy (% Recovery)	99.92-100.14
Precision (% RSD)*		
4	Intra- Day	0.250
5	Inter- Day	0.328
6	Ruggedness(% RSD)*	0.516
Robustness(% RSD)*		
7	Change in Wavelength	0.015
8	Change in Flow Rate	0.031
9	LOD^{a} µg/ml	0.68
10	LOQ^{b} µg/ml	1.05

*All the values expressed as a mean Six Determination

^{a}LOD = Limit of detection.

^{b}LOQ =Limit of quantitation.

RESULTS AND DISCUSSIONS

After extraction of Podophyllotoxin (PPT), a suitable HPLC method was developed and validated for the determination of Podophyllotoxin [TLC, R_f = 0.85] content in root extracts of different populations *Podophyllum hexandrum*. Different mobile phase combinations were tested, and according to the preliminary results, the mobile phase of acetonitrile/water (0.01% formic acid, 1N) accomplished in a gradient condition, which was able to separate the favored lignans (Figure 1). This method was comprehensively validated for its accuracy, linearity, robustness, specificity, precision and intermediate precision under various modified conditions as per ICH guidelines.

Six concentration levels of standard PPT were prepared and subjected to HPLC, and the corresponding peak areas were utilized so as to draw the calibration curves. Excellent linearity in a range of 0.10-80.0 μg/ml was achieved and the regression equations were y = 180212x-18.1816 with a correlation coefficient of 0.9997. Limit of detection (LOD) and limit of quantification (LOQ) indicate the sensitivity of method and were low (Table 1). Calculated %RSD for peak areas related to triplicate injections of the standards was found to be less than 1.5%. These results indicate that the proposed HPLC method is sufficiently sensitive for the determination and quantitation of Podophyllotoxin (PPT) at low concentrations.

Recovery test confirmed the accuracy test and after the addition of accurate amount of each standard at three levels (15, 25and 35%) to the extract, we analyzed it by the proposed HPLC method (Table 1), were close to 100% in almost all cases and this method can be considered accurate for the present research. Precision was evaluated by assaying 6 replicate injections of standards at the same concentration, during the same day and 7continuous days. The intra-day precision was <0.2500%, and inter-day precision was <0.3280% of the PPT standards (Table 1). Since the results were within the acceptable range confirm the accuracy and precision of the method.

Table 2: Content of Podophyllotoxin in Root Sampes of Podophyllum Hexandrum

RHP	54.3	5.43
CHP	36.8	3.68
SHP	23.4	2.34
KUK	24.9	2.49
DHP	22.1	2.21

In order to assess the robustness of the method, we modified several parameters, such as flow rate from 1.0 to 1.1 and 0.9 mL/min, and wavelength from 284 nm to 294 nm, and no significant changes were observed in the resolution or response of the standard peaks. The results indicated good linearity, sensitivity, accuracy, precision, specificity, and robustness of this method to be suitable for the quantitation of Podophyllotoxin (PPT) in root extracts of *Podophyllum hexandrum* collected from different sites. In order to exhibit the ability of the method for the analysis of the root extract of *Podophyllum hexandrum*, crude extract was subjected to HPLC under optimum chromatographic separation conditions (Figure 1). Moreover, for identification of the related peak of PPT, crude extract was spiked with standard PPTsolutions, and the corresponding APCI-MS/MS mass spectrum were acquired (Figure 2-4). Quantification of Podophyllotoxin (PPT) in root extracts of *Podophyllum hexandrum* were performed using calibration curves. Results of quantification of the PPT in collecting samples revealed that the concentration levels of PPT vary from one sample site to the other of plants dry weight (Table 2).

By studying, the MS pattern and molecular ion peaks as revealed in corresponding mass spectra, Podophyllotoxin and related Lignan marker compounds were identified in various extracts. The compound identification was possible on the basis of the different Molecular ionization pathways. Since the molecular mass for Podophyllotoxin is 414.405, so we found APCI-MS/MS mass of Standard Podophyllotoxin at 415.2 $[M+1]^+$. To ensure and assess the quality of PPT, product ion pairs of *m/z* 414.4 → 437.5 adduct with Sodium, *m/z* 414.4 → 432.4 adduct with water and *m/z* 414.4→ 828.8 timer PPT were observed at the molecular ion peak with highest signal abundance in their MRM mode of the APCI soft mass spectrum, using software modulations.

Correlation of Podophyllotoxin Content with Environmental Factors

After quantification of Podophyllotoxin content in root extract of *Podophyllum hexandrum*, dependence of its content on environmental variables, including altitude, and average minimum temperature were evaluated. As it is obvious from Table.2, quantification of Podophyllotoxin exhibited a direct relation between its content and the altitude of the plant collection site. According to the results, as the altitude (height from the sea level) of the plant ecosystem increased, the content of Podophyllotoxin raised in the roots of plants. As the average temperature of the plant growing site rises, a downward trend is observed for Podophyllotoxin contents in root samples. This implies that temperature has a negative effect on the content of PPT in this plant. Therefore, results indicate that inhabitants in more northerly and less westerly positions, and at higher elevations, were accompanied by higher Lignan levels. As it is proven by the previous studies, as well as variations in environmental variables, such as latitude and climate resulted in enhancement of biosynthesis of the secondary metabolites or storage of them, as a defense mechanism. Almost always, increasing the altitude is associated with lower temperatures of the plant growing site. This may shed light on the approximately similar behavior of variations in lignans contents towards higher altitudes and lower temperatures. Lower temperature is considered as an environmental abiotic stress and can cause responses in plant, in which lignans may significantly take part. Regarding the results of previous investigations as well as those of ours, we suggest that environmental variables can put substantial impact on PPT content. Higher concentrations of Podophyllotoxin in the roots of *Podophyllum hexandrum*, were associated with lower temperatures and higher altitude.

Table 3: Effect of Different Concentrations and Composition of Plant Growth Regulators for Percent Callus Formation in *Podophyllum Hexandrum*

Medium Code (MS Basal)	NAA (mg/L)	Kn (mg/L)	BA (mg/L)	2,4-D (mg/L)	Percent Callus Formation (%)
C_0	2.00	1.00	-	-	0.00 (0.00)
C_1	2.00	-	-	-	0.00 (0.00)
C_2	3.00	2.00	-	-	0.00 (0.00)
C_3	2.00	1.5	-	-	0.00 (0.00)
C_4	2.00	2.00	-	-	0.00 (0.00)
C_5	-	2.00	1.00	-	6.66 (8.85)
C_6	-	2.00	1.5	-	13.33 (17.70)
C_7	2.00	-	3.00	-	20.00 (26.55)
C_8	5.00	-	-	1.00	6.667 (8.85)
C_9	1.5	-	2.00	-	40.00 (38.83)
C_{10}	1.5	-	2.5	-	53.33 (46.90)
C_{11}	-	1.00	-	3.00	0.00 (0.00)
C_{12}	1.5	-	1.00	-	26.66 (30.77)
C_{13}	-	1.00	2.00	-	6.66 (8.85)
C_{14}	1.00	-	-	2.00	0.00 (0.00)
C_{15}	-	2.00	2.00	-	6.66 (8.85)
C_{16}	-	-	1.00	3.00	6.66 (8.85)
C_{17}	5.00	-	2.00	-	26.66 (30.77)
C_{18}	3.00	-	1.00	-	93.33 (81.13)
C_{19}	1.00	-	-	2.00	13.33 (17.70)
C_{20}	3.00	1.00	-	-	0.00 (0.00)
C_{21}	1.5	-	2.00	-	73.33 (59.18)
C_{22}	2.00	-	-	3.00	0.00 (0.00)
$CD_{0.05}$					15.37 (16.24)
SE±					5.38 (5.68)

*The values expressed in parentheses are sine transformation of percentage

CONCLUSIONS

This work proposes a validated method for quantification of Podophyllotoxin in the roots of *Podophyllum hexandrum* and for the chemical profiling of Podophyllotoxin from *Podophyllum hexandrum* developed, based on the MS finger printing of the standard marker, and could be employed in the absence of reference standards for the marker, and was particularly useful, in view of the scarcity of the chemical standard of Podophyllotoxin. Application of MRM mode to analyze the content of Podophyllotoxin from *Podophyllum hexandrum* is also presented. Besides, the data obtained from this analysis will be further employed to investigate the genotoxicity induced *by Podophyllum species.* We also conclude that environmental variables can put substantial impact on Podophyllotoxin content and higher concentrations of Podophyllotoxin were associated with lower temperatures and high altitude from sea level. The addition of precursors ferrulic acid in the suspension culture improved HPLC purity to 5.845 % at 10 µM after 8 days and of eugenol at 15 µM after 16 days it was 4.188%. Treatments of cultured cells with different CMC's with fungal elicitors have shown to induce the phenylpropanoid bio-synthetic routes (Table-3). And, electors methyl Jasmonate at 15 µM after 4 days resulted in higher HPLC purity (8.6006 %) and of isosafrole at 15 µM after 7 days shows 5.2414%.

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